ARTICLE

Stereochemical Effects in an Insect Repellent

JEROME A. KLUN,[1] WALTER F. SCHMIDT,[2] AND MUSTAPHA DEBBOUN[3]

J. Med. Entomol. 38(6): 809–812 (2001)

ABSTRACT Racemic 1-[3-cyclohexen-1-ylcarbonyl]-2-methylpiperidine repels blood-feeding arthropods such as mosquitoes, chiggers, and ticks. The compound contains two asymmetric carbon atoms and the racemate consists of four stereoisomers. Quantitative mosquito bioassays using *Aedes aegypti* (L.) showed that (1S,2′S) and (1R,2′S) configurations were 2.8–3.1 and 1.6–1.8 times more effective, respectively, than the other two stereoisomers in reducing mosquito bites. (1S,2′S) was 2.5 more repellent than the racemate. Biological data show that an interaction of the (2′S)-2-methylpiperidine configuration with a repellent receptor system in *A. aegypti* is apparently important to repellent activity. Nuclear magnetic resonance spectra and molecular mechanics calculations for the stereoisomers provided insight into the conformation of the (2′S)-group. Results indicate that enhanced repellent effects can be realized through formulation of the most active stereoisomers of the compound.

KEY WORDS *Aedes aeqypti*, structure activity relationship, chirality, repellent, nuclear magnetic resonance

RACEMIC 1-[3-cyclohexen-1-ylcarbonyl]-2-methylpiperidine was first identified as an insect repellent 23 yr ago (McGovern et al. 1978). The U.S. Department of Agriculture assigned the compound a code number, AI3–37220, hereafter referred to as 220 (Fig. 1). Although the compound has been called a repellent, it does not reduce the number of blood-sucking arthropods visiting a site where the compound is applied. However, it acts as an anti biting agent when skin is treated topically with the compound. The term repellent is used in this context. Field and laboratory studies against many species of blood-sucking arthropods have consistently shown that 220 is as, or more, effective than the commonly used arthropod repellent, deet (N,N-diethyl-3-methyltoluamide) in reducing bites (Coleman et al. 1993, Frances et al. 1996, Frances et al. 1998, Robert et al. 1992, Walker et al. 1996Debboun et al. 2000). Compound 220 contains two asymmetric centers and standard symmetric synthesis yields an equal mixture of four stereoisomers [rectus (R), sinister (S)], SR, SS, and RR. All previous studies with 220 were conducted using the stereoisomeric mixture (racemate). To date, however, the repellent properties of the compound's individual stereoisomers have not been evaluated.

The stereochemical configuration of a molecule (chirality) influences biological potency and selectivity of insect responses to natural-product and synthetic chemicals (Kurihara and Miyamoto 1998). Less active stereoisomers in mixtures dilute the potency of the more active isomers, and individual isomers can have generally different biological properties (Testa 1990). It was therefore useful to determine if chirality played a role in the known arthropod-repellent effect of 220.

It is also known that changes in conformation can directly influence the physical properties (Schmidt et al. 1988, Schmidt and Honigberg 1991) and biological activities (Jayasundera et al. 1999) of stereoisomers. Thus, consideration of structure-activity relationships for asymmetric compounds must include evaluation of both configurational and conformational aspects of the compounds. We used a mosquito bioassay to address the configurational aspects of 220 stereoisomer repellence. Nuclear magnetic resonance (NMR) spectroscopy and molecular mechanics calculations were applied to provide insight into the conformational aspects of 220 repellence.

Materials and Methods

Bioassay Chemicals. Pure 220 stereoisomers were prepared and characterized earlier (Klun et al. 2000). The stereoisomers were previously evaluated toxicologically and approved for use in entomological studies with humans (Snodgrass and Harvey 1998). In conducting this research, we adhered to the guidelines established by the National Institutes of Health for tests involving human subjects, and protocols were approved by the Human-Use Review Board of the Walter Reed Army Institute of Research. The study evaluated the blood feeding (biting) frequency of *Aedes aegypti* (L.) mosquitoes in response to six treat-

This article reports the results of research only. Mention of a proprietary product does not constitute an endorsement or a recommendation by the USDA for its use.

[1] USDA-ARS-PSI, Chemical Affecting Insect Behavior Laboratory BARC-West, Beltsville, MD 20705 (e-mail: jklun@asrr.arsusda.gov).

[2] USDA-ARS-NRI, Environmental Chemistry Laboratory, BARC-West Beltsville, MD 20705.

[3] Department of Entomology, Division of Communicable Diseases and Immunology, Walter Reed Army Institute of Research, Silver Spring, MD 20910.

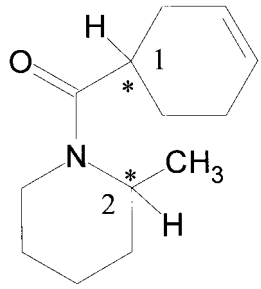

Fig. 1. Chemical structure of racemic 220. Asymmetric carbon atoms are adjacent to the numbers in the two rings that comprise 220.

ments (220 racemate, four stereoisomers and a control) applied to human skin.

Insects. Mosquitoes were reared (Hoch et al. 1995), pathogen free, at the Walter Reed Army Institute of Research. They were maintained at a photoperiod of 12:12 (L:D) h. at 27°C and 80% RH. Mated females (5–15 d old) had access only to water 24 h and neither food nor water for another 24 h before testing. Mosquitoes were used once in a test and then frozen.

Test Methods. Bioassays were conducted by using six-celled K & D modules and methods described by Klun and Debboun (2000) to evaluate arthropod repellent efficacy using human subjects. Each of six adjacent cells in the K & D modules was provided with five female mosquitoes randomly selected from cages containing ≈200 adults. A human volunteer wearing short pants was seated with legs horizontally extended. Using a skin-marking template and a water-soluble marker, skin areas representing the six floor openings of the K & D module were outlined on the outer, top, and inner thigh of each leg. A replicate consisted of the six treatments applied randomly to each of the six thigh positions. The bioassay was replicated 36 times (two volunteers with 12 replicates each and two volunteers with six replicates each).

Each treatment (1.81 mg compound/ml ethanol) was pipetted onto a 4 by 5-cm rectangular area of the subjects' skin using 55 ml ethanol/treatment. Thus, the dose of each treatment was 4.95 mg/cm^2 skin. Skin treated with ethanol alone was the control. The K & D module was positioned over the treated skin areas, and number of females biting (proboscis inserted into skin and/or observed blood-engorged females) within each of the six cells containing five mosquitoes in a 2 min skin exposure was recorded. Individual mosquitoes were scored as either having fed or not having fed during a trial. Repellent bioassays were done in a walk-in incubator (27°C and 80% RH) in ambient fluorescent light from 0730 hours to 1030 hours over 3 d. Sums of the numbers of biting and nonbiting mosquitoes (a binary response) in a group were treated as binomial data (mosquitoes were assumed to act independently), and analyzed using standard log-linear methods. A log-linear model for the data set was constructed using a stepwise procedure, in S-Plus 4 (Mathsoft 1997). The classification variables were treatment and subject and th eresponse variables were frequency of biting and nonbiting mosquitoes. To determine which pairs of treatments differed, we used the method developed by Levy (1975) to make pairwise comparisons of the proportions of biting mosquitoes. The significance level was set at $P = 0.05$.

Table 1. Comparative repellent effectiveness of 220 stereoisomers and racemate (4.95 mg compound per cm^2 skin) and untreated skin (control) against *A. aegypti* ($n = 36$)

Treatment	Total bites sustained[a]	Proportion biting[b]
(1S,2′S)	32 (5.2)	0.18a
(1R,2′S)	58 (6.3)	0.32b
(1S,2′R)	92 (6.7)	0.51c
(1R,2′R)	101 (6.6)	0.56c
Racemate	81 (6.6)	0.45bc
Control	150 (5.0)	0.83d

[a] Number in parenthesis is the standard error.
[b] Proportions followed by the same letter are not significantly different from one another at $P = 0.05$.

Physical Chemistry. NMR spectra were recorded using a Bruker QE Plus 300 MHz NMR spectrometer. Proton spectra were acquired with a spectral width of 3100 Hz and 4K data points. Chemical shifts are reported relative to tetramethylsilane. The spectra were collected at 25 ± 5°C. No line broadening was used with the free induction decay signal. Solutions (0.5 mg/ml) were prepared of the individual 220 stereoisomers in deuterated solvents (benzene-d_6, acetone-d_6, $CDCl_3$ and CD_2Cl_2). Molecular mechanics were done using MM3 (Allinger et al. 1989) in the computational chemistry program, Alchemy (Tripos, St. Louis, MO).

Results and Discussion

Bioassay results obtained with *A. aegypti* are shown in Table 1. For these data, there were no differences among subjects, but significant differences among treatments. We report the total number of bites sustained within each treatment and the proportion of mosquitoes that bit in each treatment. The data are valuable because they provide insight into the chiral nature of the 220 chemoreceptor in this arthropod. The data show that the repellent chemoreceptor system in *A. aegypti* accommodates the (2′S) moiety more effectively than (2′R). Notably, stereoisomers (1S,2′S) and (1R,2′S) proved 2.8–3.1 and 1.6–1.8 times more repellent than the other stereoisomers, respectively. The racemate and (1S,2′R) and (1R,2′R) stereoisomers had repellent activities that were not significantly different from one another. If one considers the proportion biting shown in Table 1 to represent the intrinsic repellent effect of each stereoisomer, the mean proportion biting for all stereoisomers is 0.39, and this approximates the observed proportion biting value (0.45) for the racemate. This indicates that the effects of the individual stereoisomers in the racemate are additive. (1S,2′S) stereoisomer was 2.5 times more effective in suppressing *A. Aegypti* bites than the racemate at an equal dose, and it is known that 220 race-

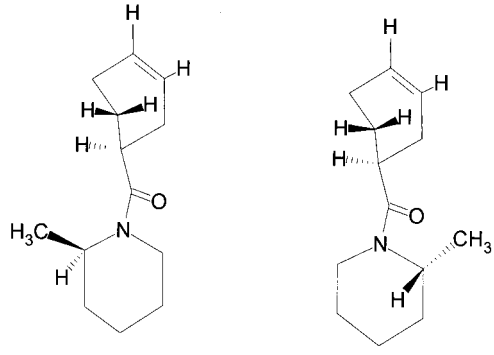

Fig. 2. Molecular mechanics model showing the two stable conformations of the (1S,2′S)-stereoisomer configuration.

mate is more effective than deet. It follows that formulations of the (1S,2′S) asymmetric 220 repellent will likely exceed the effectiveness of deet against *A. aegypti* and other blood-sucking arthropods. This is of practical significance; it will be of considerable interest to learn if field tests confirm this hypothesis. If field tests demonstrate that asymmetric 220 formulations provide enhanced arthropod protection levels, then manufacture of bulk quantities of new asymmetric repellents would be needed for commercialization. Fortunately, large scale preparations of asymmetric repellents should be entirely feasible using existing asymmetric-synthesis technology such as that developed by Vries et al. (1998).

The (2′S)-ring is a common structural feature in the most repellent isomer [(1S,2′S)-220] and the second most repellent isomer [(1R,2′S)-220]. The (1R,2′R)-220 isomer was significantly less effective as a repellent than either. This is further supportive evidence of the importance of the S-chiral configuration at the (2′)-ring on these compounds for optimal repellent activity.

The molecular structure of [(1S,2′S)-220] however can exist in two distinct stable conformations (Fig. 2). The same two conformations exist at the (2′)-ring for [(1R,2′S)-220]. Chemical structures containing six-membered aliphatic carbon rings can exist in multiple conformations (Steitwieser and Heathcock 1981). Restricted rotation due to steric hindrance at the amide moiety can deter interconversion of the two conformations. Molecular mechanics calculations predict that the two forms are equally likely and stable.

The reason [(1S,2′S)-220] has higher activity than [(1R,2′S)-220] could be the configuration of the other chiral center: the 1S site is structurally also more involved in repellent activity than the 1R site. However, we present evidence of another explanation: the conformation at the 2′S site is different between the two isomers, and propose the conformational difference at the most active (2′S)-site unambiguously can affect repellent activity.

Nuclear magnetic resonance has been used to investigate preferred conformations in solution; broad peaks are evidence of multiple stable conformations simultaneously present (Harris 1986). Broadening of peaks especially in the (2′)-ring occurred in both samples, but peaks in the less active component [(1R,2′S)-220] (Fig. 3A) were sharper than in the most active isomer (Fig. 3B). Specifically, the methyl peak on the 2′S-ring at 1.5 ppm in (1S,2′S)-220 isomer is so broad that it overlaps with the other aliphatic proton peaks from 1.2–1.6 ppm. In contrast, the methyl

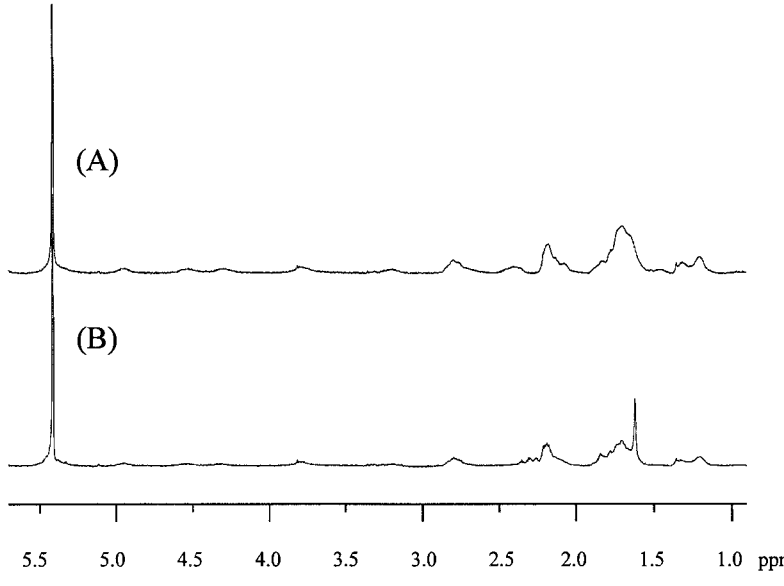

Fig. 3. NMR spectra of (1S,2′S) [A] and (1R,2′S) [B] stereoisomers of 220.

peak at 1.5 ppm is easily found in (1R,2′S)-220 isomer. Comparable results were seen independent of the solvent selected. The conformations at the (2′S)-site are more rigid, more sterically hindered in the (1R)-configuration than in the (1S)-configuration. Thus, the (2′S)-ring is required for proper interaction with the repellent receptor, and correct conformation and configuration at the 2′-chiral center are both essential stereochemical components for (1S,2′S)-220 repellent activity.

It will be of interest to learn if the magnitude of stereochemical effects seen here and the (1S,2′S) and (1R,2′S) stereochemical specificity of 220 repellence observed in *A. aegypti* generalizes to other blood-sucking arthropods or, if in other species, different stereoisomers will be more active. Hypothetically, 220-receptor binding sites responsible for repellence might not be the same in all blood-sucking arthropods. This information could be useful for designing analogous compounds with better or taxon-specific repellent properties.

Acknowledgments

We thank Dustin Stover, William Wyatt, and Jason Forguson for volunteering for the mosquito tests and Lara Gilmore for technical assistance in the testing and Matt Kramer for statistical analyses and editorial comments.

References Cited

Allinger, N. L., Y. H Yuh, and J.-H. Lii. 1989. Molecular mechanics. The MM3 force field for hydrocarbons. J. Am. Chem. Soc. 111: 8551–8566.

Coleman, R. E., L. L. Robert, L. W. Roberts, J. A. Glass, D. C. Seeley, A. Laughinghouse, P. V. Perkins, and R. A. Wirtz. 1993. Laboratory evaluation of repellents against four Anopheline mosquitoes (Diptera: Culicidae) and two Phlebotomine sand flies (Diptera: Psychodidae). J. Med. Entomol. 30: 499–502.

Debboun, M., D. Strickman, V. B. Solberg, R. C. Wilkerson, K. R. McPherson, C. Golenda, L. Keep, R. A. Wirtz, R. Burge, and T. A. Klein. 2000. Field evaluation of deet and a piperidine repellent against *Aedes communis* (Diptera: Culicidae) and *Simulium venustum* (Diptera: Simuliidae) in the Adirondack mountains of New York. J. Med. Entomol. 37: 919–923.

Frances, S. P., R. D. Cooper, and A. W. Sweeney. 1998. Laboratory and field evaluation of the repellents Deet, CIC-4, and AI3–37220 against *Anopheles farauti* (Diptera: Culicidae) in Australia. J. Med. Entomol. 35: 690–693.

Frances, S. P., T. A. Klein, D. W. Hildebrandt, R. Burge, G. Noigamol, N. Eikarat, B. Sripongsal, and R. A. Wirtz. 1996. Laboratory and field evaluation of Deet, CIC-4, and AI3–37220 against *Anopheles dirus* (Diptera: Culicidae) in Thailand. J. Med. Entomol. 33: 511–515.

Harris, R. K. 1986. Nuclear magnetic resonance spectroscopy: a physicochemical view. Longman Scientific & Technical, Essex, England.

Hoch, A. L., R. K. Gupta, and T. B. Weyandt. 1995. Laboratory evaluation of a new repellent camouflage face paint. J. Am. Mosq. Control Assoc. 11: 172–175.

Jayasundera, S., W. F. Schmidt, C. J. Hapeman, and A. Torrents. 1999. Influence of the chemical environment on metolachlor conformations. J. Agric. Food Chem. 47: 4435–4442.

Klun, J. A, and M. Debboun. 2000. A new module for quantitative evaluation of repellent efficacy using human subjects. J. Med. Entomol 37(1): 177–181.

Klun, J. A., D. Ma, and R. Gupta. 2000. Optically active arthropod repellents for use against disease vectors. J. Med. Entomol 37: 182–187.

Kurihara, N., and J. Miyamoto (eds.). 1998. Chirality in Agrochemicals. Wiley, New York.

Levy, K. J. 1975. Large-sample pair-wise comparisons involving correlations, proportions, or variances. Psychol. Bull. 82: 174–176.

McGovern, T. P., C. E. Schreck, and J. Jackson. 1978. Mosquito repellents: alicyclic amides as repellants for *Aedes aegypti* and *Anopheles quadri maculatus.* Mosq. News. 38: 346–349.

Robert, L. L., R. E. Coleman, D. A. Lapointe, P.J.S. Martin, R. Kelly, and J. D. Edman. 1992. Laboratory and field evaluation of five repellents against the black flies *Prosimulium mixtum* and *P. fuscum* (Diptera: Simuliidae). J. Med. Entomol. 29: 267–272.

Schmidt, W. F., and I. L. Honigberg. 1991. Nuclear magnetic resonance (NMR) spectroscopic investigation of interaction energies of ephedrine stereoisomers in noncrystalline solids and its correlation with thermodynamic data. Pharm. Res. 8: 1128–1136.

Schmidt, W. F., W. Porter, and J. T. Carstensen. 1988. Thermodynamics in the prediction of the solubilities of binary mixtures of the diastereoisomers and enantiomers of ephedrine. Pharm Res. 5: 391–395.

Snodgrass, H. L., and J. G. Harvey. 1998. Toxicology study No. 86–8104–98, The acute toxicity of the diastereomers of the insect repellent AI3–37220, June 1998. U.S. Army Center for Health Promotion and Preventative Medicine, Aberdeen Proving Ground, MD.

Mathsoft. 1997. S-Plus 4 guide to statistics. Data Analysis Products Division, MathSoft, Seattle, WA.

Steitwieser, Jr., A., and C. H. Heathcock. 1981. Introduction to organic chemistry, 2nd ed. Macmillan, New York.

Testa, B. 1990. Definitions and concepts in biochirality, pp. 15–32. *In* B. Holmstedt, H. Frank, and B. Testa (eds.), Chirality and biological activity. Liss, New York.

Vries, T., J. Wynberg, E. Van Echten, J. Koek, W. Ten Hoeve, R. M. Kellogg, Q. B. Broxterman, A. Minnaard, B. Kaptein, S. Van der Sluis, L. Mulshof, and J. Kooistra. 1998. The family approach to the resolution of racemates. Angew. Chem. Int. Ed. 37: 2349–2354.

Walker, T. W., I. L. Robert, A. Copeland, A. K. Githeko, R. A. Wirtz, J. I. Githure, and T. A. Klein. 1996. Field evaluation of arthropod repellents, Deet and a Piperidine compound, AI3–37220, against Anopheles funestus and Anopheles arabiensis in western Kenya. J. Am. Mosq. Control. Assoc. 12: 172–176.

Received for publication 26 October 2000; accepted 20 April 2001.